PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference HSM-LUP-GYR	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/IN 02/00192	International filing date *(day/month/year)* 20.09.2002	Priority date *(day/month/year)* 20.09.2002
International Patent Classification (IPC) or both national classification and IPC C07K16/12		
Applicant LUPIN LTD		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 3 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the opinion
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
19.04.2004	24.11.2004
Name and mailing address of the international preliminary examining authority: European Patent Office - Gitschiner Str. 103 D-10958 Berlin Tel. +49 30 25901 - 0 Fax: +49 30 25901 - 840	Authorized Officer Fuchs, U Telephone No. +49 30 25901-321

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/IN 02/00192

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:

Description, Pages

1-3, 5-12, 14-18	as originally filed
4	received on 28.07.2004 with letter of 26.07.2004
13	received on 05.11.2004 with letter of 03.11.2004

Sequence listings part of the description, Pages

1-3	as originally filed

Claims, Numbers

1-5, 7	as originally filed
6, 8	received on 28.07.2004 with letter of 26.07.2004

Drawings, Sheets

1/7-7/7	as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).

   ☐ the language of publication of the international application (under Rule 48.3(b)).

   ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   ☒ contained in the international application in written form.

   ☐ filed together with the international application in computer readable form.

   ☐ furnished subsequently to this Authority in written form.

   ☒ furnished subsequently to this Authority in computer readable form.

   ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

☐ the description, pages:

☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)).

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application,

☒ claims Nos. 1-4, 7 (partially)

because:

☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (specify):

☐ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. are so unclear that no meaningful opinion could be formed *(specify)*:

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☒ no international search report has been established for the said claims Nos. 1-4, 7 (partially)

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the Standard.

☐ the computer readable form has not been furnished or does not comply with the Standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-8
	No:	Claims	-
Inventive step (IS)	Yes:	Claims	1-5, 7, 8
	No:	Claims	6
Industrial applicability (IA)	Yes:	Claims	1-8
	No:	Claims	-

2. Citations and explanations

see separate sheet

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

As outlined in the International Search Report (ISR) the search for claims 1-4 has been restricted to those parts of the claims which appear to be clear, supported and disclosed in the sense of Articles 5 and 6 PCT, namely to the single chain antibody containing amino acid sequences SEQ ID NOS: 3 and 4 or having amino acid sequence SEQ ID NO: 2.

The same applies to the search for claim 7 which has been restricted to the monoclonal antibodies MsGyrA:C3 and MsGyrA:H11.

The International Preliminary Examining Authority fully supports the objections made in the ISR. The Applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no ISR has been established need not to be the subject of an International Preliminary Examination (Rule 66.1(e) PCT). The Applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the ISR or during Chapter II procedure.

Re Item V

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

The opinion expressed as to novelty, inventive step and industrial applicability refers only to matter for which an ISR has been drawn up.

Reference is made to the following document:

D1: MANJUNATHA, U. H. ET AL.: "Monoclonal antibodies to mycobacterial DNA gyrase A inhibit DNA supercoiling activity", EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 268, no. 7, April 2001 (2001-04), pages 2038-2046

1. Amendments (Article 34(2)(b) PCT)

The amendments filed with the letter dated 26 July 2004 are allowable, since they do not introduce subject-matter which extends beyond the content of the application as filed (Article 34(2)(b) PCT).

On page 4, line 18, the invention has been restricted to the "inhibition of DNA supercoiling activity catalyzed by *M. tuberculosis* DNA gyrases by full-length mAb and its Fab" instead of "inhibition of DNA supercoiling activity catalyzed by *M. smegmatis* and *M. tuberculosis* DNA gyrases by full-length mAb and its Fab".

The amendment of page 13, line 15, pertains to the replacement of the wording "coding" by "encoding".

Claim 6 has been corrected to pertain to "fusing said variable heavy chain and light chain regions" instead of "fusing said variable heavy chain region and light regions".

In claim 8, a functional feature ("which inhibits the activity of DNA gyrase from *M. smegmatis* and *M. tuberculosis*") of the claimed plasmid has been deleted, while it is still characterized by the technical feature "encodes an engineered single chain antibody containing amino acid sequence for inhibiting the activity of DNA gyrase from *M. smegmatis* and *M. tuberculosis*, said amino acid sequences being as shown in SEQ ID NOS: 3 and 4 respectively".

Furthermore, the amendment of page 13, line 18, of the description, submitted as corrected page 13 with the letter dated 3 November 2004 is also admissible. It clarifies the subject-matter of example E, namely the preparation of the "neutralizing single chain antibody, scFv:GyrA". Basis for the correction can be found on page 16, example E8.

2. Inventive Step (Article 33(3) PCT)

In **D1** the monoclonal antibodies MsGyrA:C3 and MsGyrA:H11 are explicitly mentioned to "form a new class of **inhibitors specific for mycobacterial DNA gyrase**" (page 2045, column 1, lines 12-14). Further, the authors state that "As the GyrA-specific mAbs described here **interact with GyrA from *M. tuberculosis*, *M. bovis*, *M. leprae* and *M. avium***, it opens the avenue to explore their potential value in the diagnosis of mycobacterial infections. It is clear that these mAbs would serve as invaluable tools to

study the enzyme in detail to address the role of DNA gyrase in the biology of mycobacteria." (page 2045, column 1, lines 33-40). Moreover, the mAbs are described to be "useful for characterizing different complexes and interactions in which DNA gyrase is involved ... (and) there is a potential to use the information to develop peptide inhibitors for DNA gyrase as a first step towards lead molecule discovery. The latter point attains considerable significance due to the alarming increase in drug-resistant **tuberculosis** in recent years" (page 2045, column 1, lines 42-48).

In the light of D1, the skilled person would expect that the inhibition of *Mycobacterium smegmatis* enzyme utilizing the said mAbs can be extrapolated to a similar inhibition of *Mycobacterium tuberculosis* DNA gyrase. Following standard methods well established in the prior art, a skilled person would make use of such mAbs in order to prepare any engineered single chain antibodies inhibiting the activity of DNA gyrase from *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*. Therefore, the subject-matter of present **claim 6** is considered to lack inventive step.

However, the preparation of the **engineered single chain antibody of present application, namely the single chain antibody containing amino acid sequences SEQ ID NOS: 3 and 4 or having amino acid sequence SEQ ID NO: 2**, is considered to be novel and inventive. In other words, **the restriction of the claimed scope to what has been actually disclosed in the description would render the objection obsolete.**

In yet another further aspect, the present invention provides an amino acid sequence of the recombinant ScFV : GyraA protein as shown in Seq. ID # 2, which inhibits DNA gyrase from *M. smegmatis* and *M. tuberculosis*.

In yet another embodiment of the invention, said engineered single chain antibody contains an amino acid sequences which inhibit the activity of DNA gyrase from *M. smegmatis* and *M. tuberculosis*, said amino acid sequences having the Seq. ID # 3 and Seq. ID # 4 respectively.

In a further aspect, the present invention provides monoclonal antibodies, viz. MSGyrA:C3 and MSGyrA:H11, which inhibits DNA gyrase from fluoroquinolone resistant *M. smegmatis* and *M. tuberculosis*.

In yet another further aspect, the present invention provides hybridoma cell lines C3B3 and H11E1, which secrete the monoclonal antibodies, MSGyrA:C3 and MSGyrA:H11, which also inhibits DNA gyrase from *M. smegmatis* and *M. tuberculosis*.

The monoclonal antibody (mAb) described in this invention has been generated against GyrA subunit of *M. smegmatis* DNA gyrase. The mAb cross reacts with GyrA subunit from fast and slow growing mycobacteria (U. H. Manjunatha et. al., *Eur. J. Biochem.*, **2001**, 268, 2038-2046). The invention describes the inhibition of DNA supercoiling activity catalyzed by *M. tuberculosis* DNA gyrases by full-length mAb and its Fab and single chain antibody (scFv) fragments. The present invention also describes inhibition of DNA gyrase activity by peptides derived from scFv. The invention also deals with novel mechanism of DNA gyrase inhibition is distinct from that of other known DNA gyrase inhibitors.

DESCRIPTION OF THE FIGURES

Figure 1A : Specificity of interaction of mAb.

Figure 1B : Effect of mAbs on mycobacterial DNA gyrase supercoiling activity.

Figure 2A : Effect of MsGyrA:C3 on DNA binding.

Figure 2B : Effect of MsGyrA:C3 on DNA cleavage.

Figure 2C : Effect of MsGyrA:C3 on ATP hydrolysis.

Figure 2D : Effect of MsGyrA:C3 on ATP independent DNA relaxation reaction of mycobacterial DNA gyrase.

Figure 3A and 3B : Effect of MsGyrA:C3 on quinolone resistant M. *smegmatis* DNA gyrase.

inhibited at 3 μg/ml and 6 μg/ml concentrations of MsGyrA:C3 for quinolone sensitive (D^S) and quinolone resistant (D^R) enzymes respectively (Figure 3B). The twofold difference in the mAb concentration between D^S and D^R enzymes is attributed to reduced specific activity of D^R enzyme. DNA gyrase from ofloxacin resistant, highly virulent clinical isolate of *M. tuberculosis* (ICC-222) was also assayed for the effect of mAb. The purified enzyme has an IC_{50} of ~10 μg/ml for ciprofloxacin, where as the MsGyrA:C3 inhibited DNA gyrase supercoiling activity at 3.0 μg/ml, similar to that of *M. smegmatis* enzyme (Figure 3C). The absence of cross-resistance essentially emphasizes the mode of action of mAb to be distinct to that of quinolones. Similar to MsGyrA:C3, MsGyrA:H11 also inhibited ciprofloxacin resistant *M. smegmatis* DNA gyrase (Figure 3D). These data confirm the novel inhibition mechanism of gyrase by mAb. Absence of cross-resistance to fluoroquinolone resistant DNA gyrase by mAb, warrants the study of MsGyrA:C3 further as it could aid in countering the drug resistance problem.

E. Cloning, sequencing and expression of a DNA sequence encoding for neutralizing antibody gene and design of bioactive peptides

This example describes the cloning and expression of a nucleic acid sequence coding for a DNA gyrase neutralizing single chain antibody, scFv:GyrA.. Based on the inhibition of gyrase by scFv:GyrA and utilizing sequence of the antibody, bioactive peptides were designed and their inhibition of mycobacterial DNA gyrase was tested.

E1 : ***Cell culture and Isolation of RNA:***

Total RNA was isolated from the actively secreting mAb:C3 hybridoma cell line. Briefly, confluent hybridoma cells ($3X10^8$) were washed with ice cold IMDM medium and total RNA was extracted using TRIzol reagent (Life technologies Inc). RNA was purified using RNeasy QUIAGEN as per the manufacturer's protocol. The quality of RNA was confirmed by electrophoresis in a 1% formaldehyde agarose gel.

E2 : ***First-strand cDNA synthesis:***

The first-strand cDNA was synthesized from total RNA using the reverse transcription reaction (RT). For annealing, 5 μg of total RNA was incubated with 0.2 μg/ml of random hexamer oligonucleotide in a 10 μl reaction volume at 70°C for 5 minutes, followed by immediate chilling on ice. The annealed mix was incubated with 1 mM dNTP and 20 Units of Moloney Murine Leukemia Virus reverse transcriptase, (M-

Claims

1. An engineered single chain antibody, which inhibits the activity of DNA gyrase from *M. smegmatis* and *M. tuberculosis*.
2. The engineered single chain antibody as claimed in claim 1 wherein it contains amino acid sequences for inhibiting the activity of DNA gyrase from *M. smegmatis and M. tuberculosis* said amino acid sequences having the Seq. ID # 3 and 4 respectively.
3. An engineered single chain antibody as claimed in Claim 1 wherein said antibody has a nucleotide sequence shown in Seq. ID # 1.
4. An engineered single chain antibody as claimed in Claim 1 wherein said antibody has an amino acid sequence shown in Seq. ID # 2.
5. A peptide having an amino acid sequence as shown in Seq. ID # 2.
6. A process for the preparation of an engineered single chain antibody which inhibits the activity of DNA gyrase from *M. smegmatis* and *M. tuberculosis*, said process comprising preparing complimentary DNA (cDNA) from the corresponding hybridoma cell lines which secretes monoclonal antibody, amplifying from said cDNA, DNA fragments encoding variable heavy chain region and light regions of said monoclonal antibody, fusing said variable heavy chain and light chain regions of said DNA fragments, cloning said fused DNA fragment in a plasmid, transforming said plasmid into *E. Coli* host strain, inducing said transformed cells to express said engineered single chain antibody and purifying said engineered single chain antibody from the induced cell lysate.
7. Monoclonal antibodies, which inhibit DNA gyrase from fluoroquinolone resistant *M. smegmatis* and *M. tuberculosis*.
8. A plasmid characterised in that it encodes an engineered single chain antibody containing amino acid sequences for inhibiting the activity of DNA gyrase from *M. smegmatis and M. tuberculosis,* said amino acid sequences being as shown in Seq. ID # 3 and 4 respectively.